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Review

Aquaporins with selectivity for unconventional permeants

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Abstract. The aquaporin protein family generally seems to be designed for the selective passage of water or glycerol. Charged molecules, metal ions and even protons are strictly excluded. Recently, particular aquaporin isoforms were reported to conduct unconventional permeants, *i.e.*, the unpolar gases carbon dioxide and nitric oxide, the polar gas ammonia, the oxidative oxygen species hydrogen peroxide, and the metalloids antimonite, arsenite and silicic acid. Here, we summarize the available data on permeability properties and physiological settings of these aquaporins and we analyze which structural features might be connected to permeability for non-water, non-glycerol solutes.

Keywords. Aquaporin, aquaglyceroporin, carbon dioxide, nitric oxide, hydrogen peroxide, ammonia, arsenite, silicon.

Introduction

Aquaporins (AQP) constitute a large family of integral membrane proteins that are present in all domains of life, ranging from archaea and bacteria to eukaryotes. They form proteinaceous pores that appear to be designed for the selective passage of water and glycerol [1].
Since the first demonstration of AQP1 from human red blood cells as a *bona fide* water channel, more than 450 individual aquaporins have been identified and an increasing number of isoforms is being functionally and structurally studied. Based on protein sequence similarity and substrate selectivity, aquaporins are grouped into two main subfamilies, *i.e.*, orthodox aquaporins, permeated exclusively by water, and aquaglyceroporins, permeated by glycerol in addition to water with the latter usually to a lower degree (see [2] for a recent review on the phylogeny of the aquaporin superfamily).
Accordingly, in unicellular microorganisms, such as *Escherichia coli*, usually two aquaporin genes are present in the genome, encoding one water channel and one glycerol facilitator [3]. However, more aquaporin genes are found in the genomes of multicellular organisms. For example, *Arabidopsis thaliana* contains 38 putative aquaporin genes [4], while the human body expresses 13 aquaporins with specific organ, tissue and cellular localization [5]. Consequently, different members of the aquaporin family can be expected to function in a diverse array of physiological processes that involve water or glycerol transport across the membrane.
The most striking feature of the aquaporin channels is perhaps their high selectivity and efficiency with regard to water or glycerol passage and the strict exclusion of ions including protons [6]. Apart from water and glycerol, a number of other permeants, *i.e.*, CO_2, NO, H_2O_2, NH_3, $As(OH)_3$, $Sb(OH)_3$, and $Si(OH)_4$ (Fig. 1) have recently been shown to pass

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specific aquaporins. Here, we summarize and discuss the current view on structural, functional and physiological aspects of aquaporins that conduct these unconventional permeants.

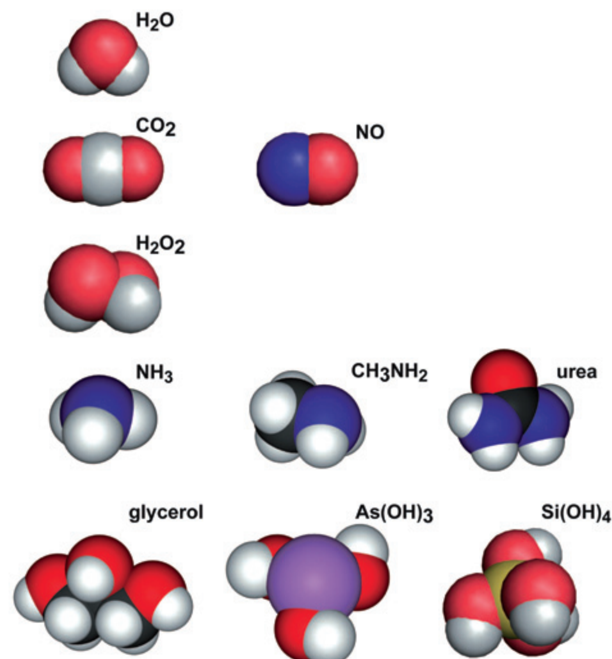

Figure 1. Molecular structures of the aquaporin permeants discussed in this review.

The general layout of aquaporin channels

Several atomic structures of aquaporins have been resolved in recent years including the mammalian aquaporins AQP0 [7–9], AQP1 [10–12], AQP4 [13], and AQP9 [14], the *E. coli* aquaglyceroporin GlpF [15, 16] and orthodox aquaporin AQPZ [17], the archaebacterial AQPM [18], and plant SoPIP2;1 [19]. The data revealed a general layout of the aquaporin channels.

All aquaporins form tetramers with each monomer functioning as an independent water pore. A monomer forms six transmembrane helices (Fig. 2a). The amino acid sequence of the second protein half is similar to the preceding sequence and thus the overall monomer is considered as formed by two imperfectly repeated parts, with each repeat containing three transmembrane helices and one short helix (called HB and HE). The two repeats are oppositely orientated such that the two short helices HB and HE meet in the middle of the membrane. Van der Waals interactions between the proline residues of two almost invariable Asn-Pro-Ala (NPA) motifs stabilize the stacking of the HB and HE short helices at the center of the water pore (Fig. 2a). Despite a common layout critical differences were identified between water channels and glycerol facilitators in two constriction regions of the channel (Fig. 2b; see [20] for a review on the structure of aquaporins).

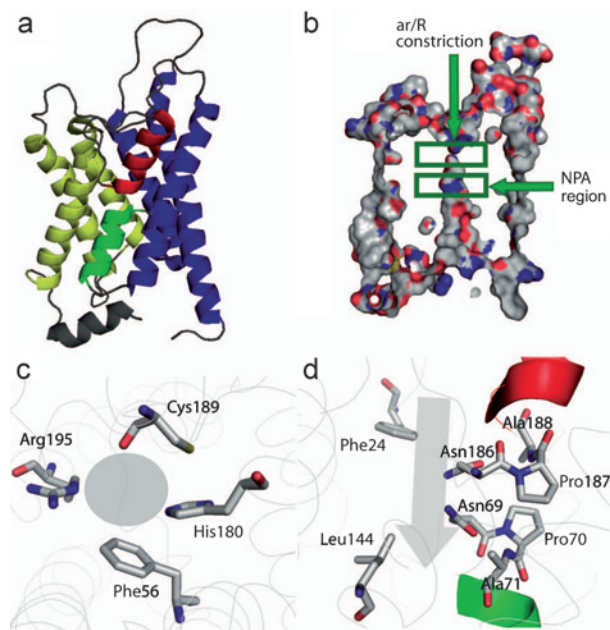

Figure 2. Various views of the prototypical aquaporin (AQP)1 crystal structure (PDB # 1J4N). (*a*) Cartoon of an AQP1 monomer as viewed from the side depicting the two repeated protein halves (blue and yellow helices) and the two short pore forming helices HB (green) and HE (red). The connecting loops are shaded in gray. (*b*) Vertical cross-section of AQP1 showing the location of the conserved aromatic/arginine (ar/R) constriction and the Asn-Pro-Ala (NPA) region. The arrows indicate the viewing direction on (*c*), *i.e.*, residues of the ar/R constriction, and (*d*), the NPA region of AQP1.

The first and narrowest constriction is located close to the extracellular pore mouth. In water-specific aquaporins it is approximately 2.8 Å in diameter, *i.e.*, identical to that of a water molecule, and about 3.4 Å in aquaglyceroporins matching the diameter of a carbon hydroxyl group of polyols such as glycerol. This constriction, referred to as aromatic arginine (ar/R) constriction, is formed by four residues, *i.e.*, Phe56, His180, Cys189, and Arg195 in AQP1 or Trp48, Gly191, Phe200, and Arg205 in GlpF. A histidine is typical for water-specific aquaporins, which together with the highly conserved arginine provides a hydrophilic edge in juxtaposition to an aromatic residue (Fig. 2c). The sulfhydryl group of a cysteine side chain extends into the pore and is the binding site for the AQP1-inhibitor $HgCl_2$ [21, 22]. The positively charged arginine is involved in proton exclusion because exchange to a valine residue results in proton leakage [23, 24]. In GlpF, and essentially in all other aquaglyceroporins, the ar/R region is more hydrophobic

than that of AQP1 due to the lack of the histidine and substitution of the cysteine by a second aromatic residue. This feature allows GlpF to efficiently conduct glycerol, small linear polyols and urea but makes GlpF a less efficient water channel [25] (Fig. 3).

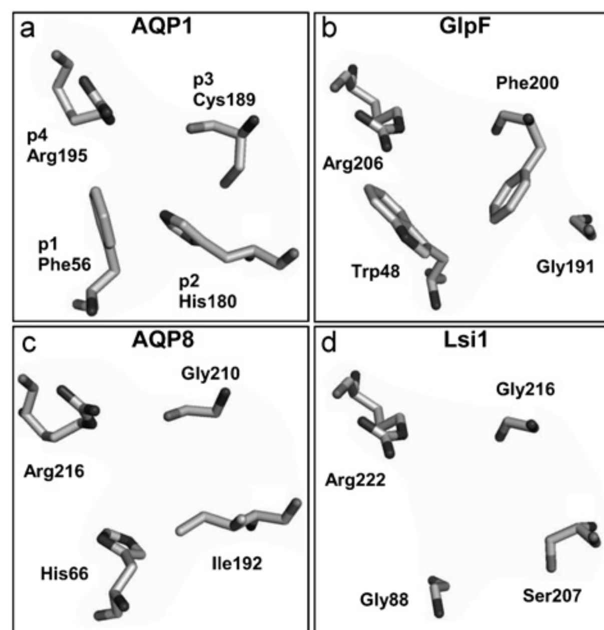

Figure 3. The ar/R constriction of four aquaporins each representing a different layout: (*a*) mammalian AQP1, (*b*) *E. coli* GlpF, (*c*) mammalian AQP8, (*d*) rice Lsi. The positions of the four residues that form the constriction are labeled p1–p4.

The second constriction is located at the center of the pore in the NPA region. The two asparagines are the capping amino acids at the positive ends of helices HB and HE and act as hydrogen donors to the oxygen atoms of passing permeants. Water that enters this region is re-oriented such that hydrogen bonds between neighboring molecules in the water chain are disrupted. This mechanism prevents the formation of a proton wire throughout the pore and represents a major energy barrier for proton conductance [6]. In GlpF, the NPA region similarly helps to block protons from passing. Selectivity by size may also be of relevance in this constriction, because almost all the aquaglyceroporins have two leucine residues opposite the two asparagines instead of a leucine and a phenylalanine in water selective aquaporins. This combination apparently results in a somewhat larger pore diameter that is suitable for solutes larger than water (Fig. 2c).
The remaining part of the aquaporin pore is lined by hydrophobic residues that expose main-chain carbonyl oxygens to the pore surface. These oxygens distribute as a ladder along one side of the pore and serve as hydrogen bond acceptor sites to efficiently funnel small hydrogen bond donor molecules, such as water, urea or polyols, through the aquaporin. Formation of hydrogen bonds between the aquaporin protein and the permeant also compensates for the energy cost due to breakage of hydrogen bonds when a molecule from the bulk solution is isolated when entering the pore. This poses the question whether appropriately sized molecules that are not hydrogen bond donors have significant permeability through AQP1.

Carbon dioxide permeability of AQP1

Carbon dioxide (CO_2) is a neutral linear molecule with a diameter similar to that of water. In 1998, Nakhoul *et al.* [26] reported the first experiment on CO_2 conductivity in AQP1-expressing oocytes. In their system, the CO_2 influx was measured using microelectrodes to monitor changes in the intracellular pH (pH_i). When the oocytes were co-injected with cRNA for AQP1 and carbonic anhydrase, an enzyme catalyzing the formation of H_2CO_3, the CO_2-induced drop in pH_i was 40 % faster than in control oocytes. The authors concluded that AQP1 expression increased the CO_2 permeability of oocyte membranes [26].
A similar strategy was employed for measuring CO_2 permeability of AQP1 in proteoliposomes with entrapped 5,6-carboxyfluorescein and carbonic anhydrase [27]. Presence of purified AQP1 in the proteoliposomes increased both permeability for water (P_f) and for carbon dioxide (P_{CO2}), approximately fourfold as compared to pure liposomes. Addition of $HgCl_2$ inhibited permeation of both; the effect could be reversed with β-mercaptoethanol due to reduction of the involved sulfur bond between mercury and Cys189. In an inward-directed CO_2 gradient of 100 mM, the permeability rate for CO_2 was calculated as 120 000 molecules/s per AQP1 subunit.
However, using AQP1 knockout mice as a physiological model to study CO_2 permeability did not confirm a role of AQP1 in CO_2 transport in erythrocytes and lung [28]. Although erythrocytes from wild-type mice exhibited a sevenfold higher osmotic water permeability than that from null mice, the CO_2 permeability, as measured from intracellular acidification using a cytoplasmic fluorescence pH indicator, of both sets of mice were not significantly different, nor was CO_2 exchange in the lung. Similar, the authors found that in AQP1-reconstituted proteoliposome experiments the presence of AQP1 did not affect CO_2 permeation [28]. This posted a controversial debate on whether an involvement of AQP1 in CO_2 transport is physiologically relevant [29, 30]. In molecular dynamics simu-

lations, the free energy profiles for CO_2 permeation displayed a barrier of approximately 23 kJ/mol in the ar/R constriction region of AQP1, but only 4 kJ/mol in a protein-free palmitoyloleoyl-phosphatidylethanolamine (POPE) lipid bilayer membrane [31]. That would argue that a contribution by AQP1 to transmembrane CO_2 permeability might only be relevant in special membranes with low intrinsic CO_2 permeability. In general, proteins are abundant in cell membranes suggesting that computer simulation data obtained from pure POPE membranes are not directly transferable to living cells [32]. The membrane of human red cells carries 140 000 copies of AQP1 and a recent study seems to indicate a role of AQP1 in CO_2 transport [33]. ^{18}O-labeled CO_2 was used to determine the exchange rates of CO_2, HCO_3^- and H_2O between the extracellular and the intracellular space of Colton null ($AQP1^{-/-}$) human red cells. The data indicated that the membranes of AQP1 null cells were permeated by CO_2 60 % slower than normal red blood cells. In plants, the physiological relevance of aquaporin-mediated CO_2 permeation seems more evident. As the substrate for photosynthetic carbon fixation intracellular CO_2 directly determines the net photosynthesis rate, which can be used as a clear system read-out. Indeed, 50-fold overexpression of *Nicotiana tobacco* AQP1 (NtAQP1) raised the net photosynthesis rate of the plants 1.4-fold in a normal atmosphere (0.038 % CO_2) and 1.8-fold in an atmosphere with elevated CO_2 levels (0.082 % CO_2), suggesting a direct coupling of NtAQP1 expression levels and CO_2 uptake [34].

Evidence for nitric oxide permeability of AQP1

Like CO_2, nitric oxide (NO) is a physiological gas and further serves as cellular signal. The diameter of NO is close to that of water; hence, passage of NO through the water pore seems biophysically plausible. Evidence for human AQP1-mediated NO permeation was recently published [35]. The rate of NO influx into CHO-K1 cells, as indicated by an intracellular NO-specific fluorescent dye, was found to correlate with expression levels of AQP1. Inhibition of AQP1 by 20 μM $HgCl_2$ reduced the NO uptake by 71 %. Variation of the NO concentration in the bathing medium showed that the uptake was saturable within a range from 0.1 to 5 μM, resulting in a K_m of 0.54 μM. The findings were underscored by assays with reconstituted proteoliposomes containing purified AQP1 that exhibited a 316 % increase in NO influx and by AQP1 siRNA knockdown experiments in cultures of pancreas endothelial MS1 cells that decreased the NO release by 44 % [35].

With the finding that AQP1 conducts gaseous molecules in addition to water, it can no longer be said to be completely water specific. This commenced the search for more aquaporin permeants among the small physiological molecules.

AQP1 does not conduct hydrogen peroxide but AQP8 does

Hydrogen peroxide (H_2O_2) molecules display a dipole moment of 2.26 D, which is slightly greater than that of water molecules (1.85 D). H_2O_2 is a weak acid with strong oxidizing properties, which is generated as one of the reactive oxygen species (ROS) during metabolic processes especially in mitochondria and in chloroplasts. It has recently been suggested that ROS may act as cellular signals. It remained unclear, however, whether H_2O_2 movement across the plasma membrane is by simple diffusion or protein-mediated. A recent study addresses this question. Bienert *et al.* (2007) expressed a panel of 24 aquaporins from animals and plants in yeast strains with different sensitivity to oxidative stress and monitored cell growth in the presence of external H_2O_2 [36]. Of the tested aquaporins, only the "orthodox" human AQP8 and the so-far water-specific *Arabidopsis* aquaporins TIP1;1 and TIP1;2 led to significantly depressed growth; AQP1 expression did not have an effect. The entry of ROS into the yeast cells was visualized using an H_2O_2-reactive fluorescent dye. Both yeast growth depression and H_2O_2-induced fluorescence could be inhibited by the addition of silver ions at 30 μM, which block AQP8, TIP1;1 and TIP1;2 by the same mechanism as mercury ions.
An interesting coincidence is that another non-water permeant, *i.e.*, ammonia, is conducted by AQP8, whereas AQP1 seems impermeable for this polar gas.

Ammonia permeability by Amt/Mep/Rh transporters and aquaporins

Ammonia in its uncharged form (NH_3) is a dipolar molecule with a trigonal pyramid shape. It readily dissolves in water and acts as a base or proton acceptor with a pK_a of 9.23. In a physiological setting at pH 7, about 99 % of the NH_3 molecules become protonated to form ammonium (NH_4^+) ions. Ammonia is a major nitrogen source for many microorganisms and plants, whereas in vertebrate animals it acts more like a cellular acid-base regulator. It is also a byproduct of nitrogen metabolism, *e.g.*, during amino acid breakdown. Accumulation of intracellular ammonia is toxic. Hyperammonemia, *i.e.*, elevated ammonia

levels in the blood, is characteristic for liver dysfunction or defects in the enzymes that convert ammonia into urea for excretion.
A family of membrane proteins (Amt/Mep/Rh superfamily) has been identified in plants and microorganisms that is responsible for the high-affinity uptake of extracellular charged NH_4^+ [37–39]. The family also includes the distantly related Rhesus (Rh) glycoproteins from mammals. Their role in mammalian NH_4^+ transport, however, is less clear.
Already before aquaporins entered the nitrogen transport scene, there was evidence that Amt/Mep/Rh proteins were not the only players in ammonia permeability across the membrane. It has been well established that ammonia influx in plant roots exhibits biphasic uptake kinetics: at external ammonia concentrations below 1 mM the uptake follows Michaelis-Menten kinetics, whereas at higher external ammonia levels (up to 50 mM) the influx becomes linear and unsaturable ([40] and references therein). Similar transport mechanism exists in microorganisms, such as *Saccharomyces cerevisiae.* Deletion of all three endogenous high-affinity ammonium transporters (Mep1–3) renders the yeast cells unable to grow on medium with low ammonia availability as the sole nitrogen source, but at ammonia concentrations higher than 5 mM the null mutant's growth is not affected [41]. Further, data indicated that pathways exist for ammonia transport in the opposite, outward direction that are also independent from Amt/Mep/Rh transporters as shown for yeast [41] and plants [42].
These results raised the question whether the low-affinity, unsaturable ammonia permeation is by simple transmembrane diffusion or by protein facilitation. First hints indicating protein involvement were derived from legume–rhizobia symbiosis within root nodules. Rhizobia bacteria reduce atmospheric N_2 to ammonia as a nitrogen supply for the plant; in return they receive organic acids as a carbon and energy source. Using isolated membrane vesicles from the peribacteroid membrane, Niemietz *et al.* [43] showed that the low-affinity ammonia permeability can be inhibited by $HgCl_2$. The mercury inhibition of ammonia permeability correlated with that of water permeability, suggesting that nodulin 26, *i.e.*, an aquaporin of legume, mediates ammonia across the peribacterial membrane.
Functional complementation in a yeast strain that is devoid of all the three endogenous ammonium transporters (Δmep1–3) has been used as a screening tool to identify ammonia conducting membrane proteins. Using this strategy, Jahn *et al.* [44] found that three aquaporins from wheat roots (TaTIP2;1–3) were able to restore ammonia uptake and growth of the yeast mutant. The complementation was pH dependent with progressively better growth at increasing pH, indicative of neutral NH_3 conductance rather than of charged NH_4^+.
Facilitation of ammonia is certainly not a general feature of orthodox aquaporins because in similar experiments mammalian AQP1 did not enhance ammonia conductance, whereas AQP8 did [44, 45]. Structurally, there are apparent similarities shared between AQP8 and TaTIP2 in the ar/R region (Fig. 3), which are absent in AQP1. We analyze such relations in a later section of this review. Suffice to say here that the ar/R constriction of the aquaporin pore seems to be directly involved in ammonia selectivity. We were able to induce ammonia permeability in AQP1 by selective point mutations in the ar/R constriction. We generated four single and double mutants by replacing Phe56 and His180 with alanine and Arg195 with valine. All mutants conducted ammonia to a similar extent as AQP8 [23]. These AQP1 mutants were originally designed to successively enlarge the diameter in the ar/R constriction and to enhance hydrophobicity with the aim to approach the situation that is present in aquaglyceroporins. Quite suggestive, all aquaglyceroporins tested so far, *i.e.* yeast Fps1, *Plasmodium falciparum* PfAQP, *Toxoplasma gondii* TgAQP and three *Trypanosoma brucei* TbAQPs, were found to conduct ammonia [23, 46].
Using methylamine, a cytotoxic analog of ammonia, and the yeast mutant BY4742Δfps1 with deletion of the single endogenous aquaglyceroporin Fps1 strongly increased the sensitivity of the assay system for aquaporin-mediated ammonia permeability [23]. In this setting, complementation of the yeast mutant relies on facilitated methylamine efflux instead of ammonia influx. Expression of PfAQP in this system rendered the yeast cells significantly less sensitive to methylamine and raised the IC_{50} by two orders of magnitude [46]. Methylamine release was found to be pH dependent, *i.e.*, lowering the medium pH generated a steeper outward-directed gradient of neutral methylamine, whereas the concentration of charged methylammonium basically remained unchanged [23]. This supports the assumption that aquaporins conduct electroneutral NH_3 rather than NH_4^+.
Due to the wider pore layout, aquaglyceroporins generally have access to a larger set of potential permeants. Indeed, toxicologically and physiologically relevant metalloids were found to pass several aquaglyceroporins.

Antimonite, arsenite and silicon permeability

In an *E. coli* mutagenesis screen on medium containing toxic metalloids, GlpF was shown to be responsible

Table 1. Connection of the discussed permeants with aquaporin isoforms and residues in the aromatic/arginine (ar/R) constriction (compare to Fig. 3) as well as important remaining pore-lining residues. Amino acids in the NPA region are boxed (see Fig. 2d).

Permeant	AQP	ar/R constr. p1 p2 p3 p4	Remaining pore-lining residues.
CO_2	AQP1	F H C R	F V I A G A H L N L V I V G G I N
	NtAQP1	F H C R	F L I V G G H I N L V I V G G I N
NO	AQP1	F H C R	F V I A G A H L N L V I V G G I N
H_2O_2	AQP8	H I G R	F I L I G G H F N L V I V G C M N
	TIP1;1 & 1;2	H I A V	F V L V G G H V N L V I V G S M N
NH_3	TIP2;1	H I G R	F V L V G G H V N L V I V G S M N
	AQP8	H I G R	F I L I G G H F N L V I V G C M N
	AQP1$^{(H180A)}$	F A C R	F V I A G A H L N L V I V G G I N
	AQP1$^{(R195V)}$	F H C V	F V I A G A H L N L V I V G G I N
	AQP1$^{(H180A/R195V)}$	F A C V	F V I A G A H L N L V I V G G I N
	AQP1$^{(F56A/H180A)}$	A A C R	F V I A G A H L N L V I V G G I N
	Fps1	W N T R	M I V Y G A H L N L T M I G A M N
	PfAQP	W G F R	L M V I G A H L N L I V I G A L N
	TgAQP	V V A V	L V V T G A H F N L C I A G T M N
	TbAQP1	W G Y R	L L V L C G H L N L V V V G A I N
	TbAQP2	I V L L	L I V L G G H L N L V I V P A M N
	TbAQP3	W G Y R	L L V L S G H L N L V V V G A I N
Sb/As$(OH)_3$	GlpF	W G F R	L I V I G A H L N L I I I G A M N
	Fps1	W N T R	M I V Y G A H L N L T M I G A M N
	AQP7	F G Y R	L M V I G A H M N L I I V G A I N
	AQP9	F A C R	L I V I G G H I N L V I I G A M N
$Si(OH)_4$	Lsi	G S G R	L V V I G A H M N M T V V G S M N

for the uptake of the toxic antimonite Sb(III) [47]. Deletion of the fps1 gene in *S. cerevisiae* rendered the yeast cells more resistant to antimonite as well as to arsenite. Accumulation of ^{74}As-labeled arsenite by Δfps1 yeast cells was 20–30 % lower than by wild-type yeast [48]. Trivalent antimonite and arsenite have a pK_a of 11.8 and 9.2, respectively. Hence, at physiological pH these metalloids most likely pass aquaglyceroporins as neutral hydroxides $Sb(OH)_3$ or $As(OH)_3$, which are structurally similar to glycerol [49]. It fits well into the picture that the mammalian aquaglyceroporins AQP7 and AQP9 also pass Sb(III) and As(III) [50].

Silicon is a trace element for animals; some plants, such as rice, however, accumulate silicon in higher amounts, which is beneficial for growth and disease resistance [51]. Silicon has been shown to be absorbed by plants and marine diatoms in the form of uncharged silicic acid, $Si(OH)_4$, and is ultimately irreversibly precipitated in the organisms as amorphous silica or silica gel (SiO_2–nH_2O). A rice mutant called *lsi1* (low silicon rice 1) is defective in silicon uptake, accumulates less silicon in the shoot during growth and shows significantly reduced disease resistance and grain yield. Map-based cloning identified the defect *lsi1* gene as a nodulin 26-like aquaporin with a single amino acid substitution A132T, which is supposed to cause a conformational change of helix 1 in the channel, rendering the protein impermeable for silicon [52]. Knock-down experiments with RNAi in rice plants led to a 5-fold reduction in silicon uptake; expression of Lsi1 in *Xenopus* oocytes increased the uptake rate of an analogous tracer $^{68}Ge(OH)_4$ 2.4-fold [52].

Obviously, $Si(OH)_4$ with a molecular diameter of 4.38 Å is larger than the diameter of the ar/R constrictions in the prototypical AQP1 and GlpF. Indeed, in Lsi1 this constriction site is differently designed and contains residues with short side chains that augment the pore diameter (Table 1, Fig. 3). This poses the question whether analysis of the constriction-forming amino acids and the pore-lining residues allows one to predict permeability properties of aquaporins.

Prediction of aquaporin selectivity from protein sequence analysis

Although the permeants discussed above are highly variable, ranging from unpolar gases to hydrogen bond donor/acceptor metalloids, they pass aquaporins

as electroneutral substrates. Further, aquaporin permeability for unconventional substrates must be seen as an additional function besides water or glycerol passage. Therefore, one can assume that the general aquaporin fold and the mechanisms for repelling ions are conserved in these particular isoforms (reviewed in [6]).

To achieve permeability for unconventional substrates, the following structural domains of the aquaporin protein may be considered: (i) residues that contribute to the vestibules on both sides of the channel; these residues are located in the most variable parts of the aquaporins, *i.e.*, the connecting loops, and may affect the entry of the substrates. Glu125 in the connecting loop C of PfAQP may serve as an example because mutation of this amino acid modulates the water/glycerol permeability ratio of this aquaglyceroporin [53]. However, predictions from simple sequence comparisons in these regions are extremely difficult due to the low degree of conservation; (ii) residues that form the ar/R constriction; (iii) residues in the NPA region, and (iv) the remaining pore-lining residues. Respective amino acids (ii–iv) from the aquaporins covered in this review are listed in Table 1.

The finding that CO_2 and NO pass through AQP1 should imply that virtually all AQP and aquaglyceroporins, regardless of their amino acid composition, are permeated by these small neutral gases. CO_2 and NO do not interact with the protein *via* hydrogen bonds or electrostatic effects and they are small enough to pass through even the narrowest aquaporin pores (Fig. 3). Of course, confirmative experiments are needed to test whether additional aquaporins besides AQP1 and NtAQP1 conduct CO_2 and NO and to clarify the physiological relevance of such permeability.

The conditions that enable H_2O_2 permeability in aquaporins seem more delicate. As mentioned, only 3 out of 24 tested aquaporins, *i.e.*, AQP8 and the TIPs 1;1 and 1;2, were found to conduct H_2O_2 [36]. These isoforms share a peculiarity with respect to the position of the histidine in the ar/R constriction, which is located at the p1 position instead of p2 (Fig. 3 and Table 1). In AQP8, His66 is thus situated next to Arg216 rather than across the constriction. In addition to an unusual positioning of the histidine, the TIP1 isoforms depict another rare substitution in the ar/R constriction, *i.e.*, the arginine itself is replaced by a valine. Comparison with other aquaporins shows that the switch in the histidine position alone cannot explain H_2O_2 permeability of AQP8 and the TIP1 proteins, because another branch of the TIP protein family, *i.e.*, the TIP2 channels, also have a histidine at p1. In fact, the ar/R constriction is identical in TIP2 proteins and AQP8 (Table 1). Yet, none of the TIP2 isoforms were found to conduct H_2O_2 in the cited study [36]. Analysis of the remaining pore-lining residues is not enlightening either. Only four conservative substitutions exist between AQP8 and TIP2;1 (Table 1), which do not justify assumptions about major differences in the pore selectivity properties. This would leave only the vestibules as selectors for H_2O_2. Obviously, more experimental data are needed to elucidate the structural determinants of H_2O_2 selectivity in aquaporins.

More consistent with sequence similarity, both AQP8 and the TIP2 proteins were identified as NH_3 channels [44, 45]. Other orthodox aquaporins such as AQP1 do not pass NH_3, indicating that for NH_3 permeability the position of the histidine in the ar/R constriction is relevant. One may interpret this situation in the ar/R constriction such that a hydrophobic edge across from the arginine-histidine pair accommodates NH_3 conductivity. This is further corroborated by the NH_3 permeability of AQP1 mutants where either the arginine or the histidine was replaced by a short hydrophobic amino acid [23]. Finally, aquaglyceroporins, which inherently posses amphipathic ar/R constrictions were found to be permeable for NH_3. These data together let us confidently predict that aquaporins with hydrophobic residues in juxtaposition to the arginine in the ar/R region, such as AQP8-like orthodox aquaporins and all aquaglyceroporins, pass NH_3.

Aquaglyceroporins even in the prototypical ar/R configuration as in *E. coli* GlpF are permeated by the metalloids $Sb(OH)_3$ and $As(OH)_3$. Due to similar dimensions and the presence of three hydroxyl groups, discrimination from glycerol is probably impossible, which would render all aquaglyceroporins permeable for Sb(III) and As(III). To accommodate the larger $Si(OH)_4$, however, a larger ar/R constriction is needed. In Lsi1, this is accomplished by an ar/R constriction layout consisting, apart from the conserved arginine, of three small residues, *i.e.*, two glycines and one serine (Fig. 3, Table 1). Whether the presence of the hydrogen-bond donor/acceptor site at Ser207 is required for the passage of $Si(OH)_4$ demands experimental confirmation.

Conclusion

What renders an aquaporin permeant unconventional? From the perspective of an aquaglyceroporin, permeability for NH_3 and metalloids does not seem unconventional at all but rather a general property. However, in the case of even larger solutes, such as $Si(OH)_4$, permeability may be a peculiarity. Similarly,

passing unpolar gases, although at a rather low level, might not be special even for orthodox aquaporins. It seems to be just a matter of time that other small neutral molecules, such as H_2S, will be identified as aquaporin permeants, because solute permeability is obviously mainly determined by the mere availability in the physiological environment. Together, it appears that development of selection mechanisms against protons and other ions was far more crucial during aquaporin evolution than selection against unwanted neutral solutes.

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